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Breeding site fidelity, and breeding pair infidelity in the endangered Carnaby's Cockatoo *Calyptorhynchus latirostris*

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Abstract

The ecology and behaviour of the endangered Carnaby's Cockatoo *Calyptorhynchus latirostris* have been studied in detail at Coomallo Creek in the northern wheatbelt of Western Australia from 1969 until the present. Results of research on this breeding population conducted on individually marked birds from 1970 to 1990 were compared with results from analyses of DNA taken from nestlings in the study area from 2003, 2005, and each year from 2009 to 2013. Analyses of DNA confirmed earlier findings about the stability of adult breeding pairs, and that females used the same breeding hollow they used previously, provided the hollow was not occupied when they returned to breed. When moving to another hollow, they chose a hollow in the same vicinity of the previous hollow. Analyses in 22 cases where DNA was obtained from both nestlings of a breeding attempt revealed that in six (27.3%) cases, the second egg was fertilised by a male not paired with the female. These extra-pair copulations were not suspected during the earlier study based on observations of individually marked birds.

Keywords

Carnaby's Cockatoo, *Calyptorhynchus latirostris*, nestling DNA, extra-pair copulations, pair bond stability, nest site fidelity

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Introduction

Carnaby's Cockatoo *Calyptorhynchus latirostris* is endemic to south-western Australia. Formally common throughout its range, over the second half of the last century, extensive clearing of native vegetation for the development of broadscale agriculture and urban development saw the species contract significantly in range and abundance. As a result of the loss of breeding and foraging habitat (Fig. 1), the status of the species changed from being classified legally up to the 1960s as vermin with a bounty on its bill, to being listed as endangered (Saunders 1990) by three authorities in the late 1980s. Currently Carnaby's Cockatoo is listed: as "Fauna that is rare or likely to become extinct" in Schedule 1 of the Western Australian *Wildlife Conservation Specially Protected Fauna Notice 2016* under the *Wildlife Conservation Act 1950*; endangered under the Australian Federal Government's *Environment Protection and Biodiversity Conservation Act* 1999; and endangered under IUCN's Red List category and criteria (IUCN 2014). IUCN recently changed the scientific name of the species to *Zanda latirostris*, but we retain the name *Calyptorhynchus latirostris* as specified in Western Australian Government notices. Since 2002 the species has been the subject of two recovery plans (Cale 2003, Department of Environment and Conservation 2012).

Beginning in 1968 and continuing, the ecology and behaviour of the species has been the focus of one of the longest running vertebrate studies in Australia (Saunders 1982, Saunders and Ingram 1998, Saunders and Dawson 2017). One breeding population of the species at Coomallo Creek (Fig. 1) was studied intensively from 1969 to 1977, then monitored intermittently using the same protocols until 1996, and then every year from 2009 (Saunders and Dawson 2017) until the present. As a result of this study, the breeding biology of the species is well known. Once the birds form pairs and reach breeding age (usually at four years), the pairs remain together until one of the partners dies. They return to the same breeding area each year in winter, with rainfall in the first half of autumn influencing the commencement of egg-laying; the wetter the period, the earlier breeding commences. The female selects a nesting hollow in a large eucalypt, usually lays two eggs, on average up to eight days apart. Both eggs usually hatch with the younger nestling dying within 48 hours of hatching. In around six per cent of cases both nestlings are successfully fledged. The female nests in the same hollow used the previous breeding season, provided she successfully fledged a young the previous year, and the hollow is not already occupied when she returns to breed. If she has an unsuccessful breeding attempt she usually moves to another hollow nearby, and makes another breeding attempt, either during the same breeding season or next season (information from Saunders 1982, Saunders et al. 2013, 2014a).

The fact that Carnaby's Cockatoo is rare, and difficult to breed in captivity (Saunders 1976) made it a lucrative target for poachers who took nestlings illegally from hollows used by wild breeding pairs. In accessing the hollow floor, poachers often cut a large hole in the side of the nesting tree rendering the hollow unsuitable for Carnaby's Cockatoo. In dealing with the problem posed by poachers, the Western Australian Government authority responsible for conservation, in conjunction with staff

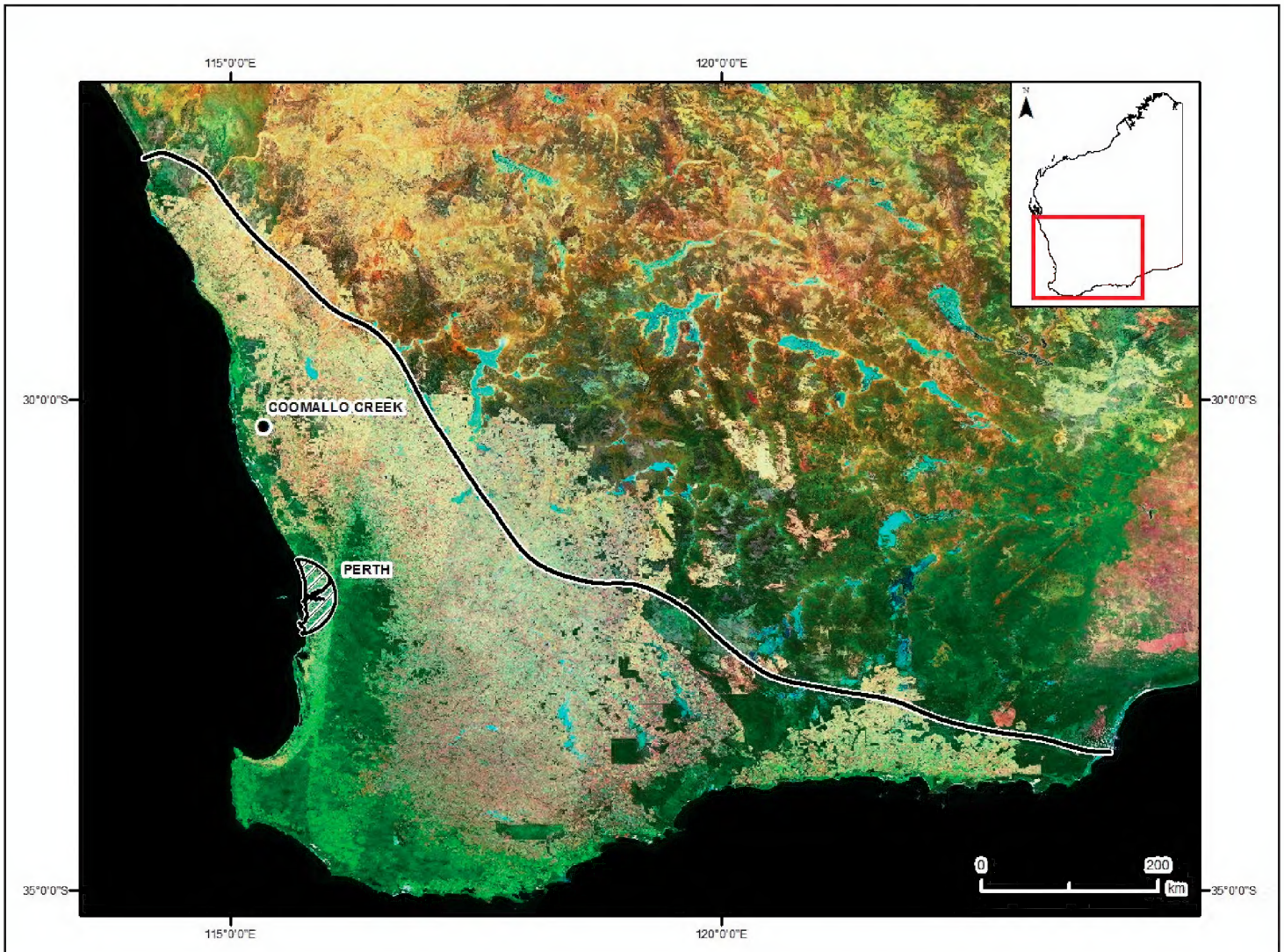

Figure 1. Location of Coomallo Creek study area, and distribution of Carnaby's Cockatoo (between the coast and the black line). Carnaby's Cockatoo has significantly decreased in range and abundance as a result of loss of breeding and foraging habitat. The extent of the loss of habitat (native vegetation indicated in pale yellow) is illustrated clearly in this satellite image.

at Curtin University, investigated the efficacy of using analysis of DNA to establish relationships between putative parents and the nestlings poachers claimed they had bred in captivity. White et al. (2009) developed 20 microsatellite markers for population and forensic applications suitable for Carnaby's Cockatoo. White et al. (2012) demonstrated the successful application of these markers in three case studies, one of which demonstrated clearly that a Carnaby's Cockatoo nestling claimed to have been bred in captivity bore no relationship to the pair the breeder claimed had produced the nestling, and further that the nestling was a full sibling of a nestling known to have been fledged successfully from a nest hollow monitored in the wild. This unequivocal result was used in a successful prosecution for poaching.

White et al. (2012) demonstrated that analyses of DNA provided reliable discriminatory power for identification of individuals, testing kinship and identification of breeding populations, making possible the determination of illegal trade or harvest of live animals from the wild, and providing a method to monitor both wild and aviary populations. They pointed out that use of DNA is a minimally-invasive technique, even less so when moulted feathers are used as described by Hou et al. (2018). White

et al. (2012) also noted that the black cockatoo database they described constituted one of the most extensive Australian wildlife forensic databases.

In this paper we examine the use of DNA taken from nestlings of one breeding population over seven breeding seasons (2003, 2005, 2009-2013) to investigate aspects of the breeding biology of Carnaby's Cockatoo. We compare the results obtained from DNA analyses with earlier research (1969-1996) on the population using observations of individually marked birds to test the efficacy of analyses of nestling DNA for studies of breeding behaviour.

Methods

The Coomallo Creek (Fig. 1) study area is described in detail in Saunders (1982), Saunders and Ingram (1998), and Saunders et al. (2015). The cockatoos nest in hollows in large eucalypt trees that are distributed along a 9km narrow belt of woodland surrounded by agricultural land and uncleared Kwongan (native shrub land *sensu* Beard 1984). There was no further clearing of the eucalypt woodland during the study period, but the number of available nest hollows fluctuated as trees were lost through natural attrition (Saunders et al. 2014b), and new hollows entered service, and were located by us.

From 1969 to 1996 the area was visited for 21 of those years for at least one week in early September, and one week in early November. During each visit every known nest hollow was inspected, and the identity of breeding females established if possible by trapping or by using a telescope to read the number on the bird's leg band/ring. Both methods were time consuming, and it was not possible to identify all banded females. Searches were also undertaken to find any nesting hollows in use not registered in the study area. Any nestlings older than three weeks were measured (length of the folded left wing and body mass) and banded with a stainless steel band with a unique number. Nestlings were aged using the methods of Saunders (1986), and laying dates extrapolated from the age of the nestlings.

The area was visited once in November 2003 and 2005, and each year from 2009 to 2013 the area was visited for one week in early September and early November, with a three-day visit in early January to establish nesting success, and band any nestlings laid late in the season. The identity of breeding females was established by photographing their legs, and checking for bands using the methods of Saunders et al. (2011). This was a much more successful method for establishing the identity of banded females nesting in the area than was possible in the earlier study. Searches were also undertaken to find any nesting hollows in use not registered in the study area. Nestlings were measured (length of the folded left wing and body mass) and banded, and laying dates extrapolated from nestling age. Nestlings were sexed on shape and colour of their cheek patch, and at least three pin feathers taken from their chests for subsequent DNA analysis. The body of small dead nestlings were taken for DNA analysis, as was at least one toe from larger dead nestlings or dead adults.

Material taken for DNA analysis was plucked with tweezers or cut with scissors, and placed in a vial of preservative (20% dimethylsulphoxide saturated with sodium chloride). The vial was labelled with species identification, collection date, name of collector, name of the study area, nest hollow number, and the description of material taken (e.g., 4 feathers, small dead nestling, toe of dead nestling). All equipment used for handling DNA was washed in disinfectant, 90% ethanol, and rinsed with water between samples to ensure no cross-contamination of DNA.

A total of 309 DNA samples were collected from Coomallo Creek between November 2003 and October 2013 for DNA extraction with Qiagen Blood and tissue kit (Qiagen). Samples were subjected to a real-time quantitative PCR (qPCR) assay to assess the DNA extracts for quality and quantity of nuclear DNA prior to microsatellite genotyping. Genetic profiles were generated from 16 microsatellite markers as previously published by White et al. (2009, 2012, 2014).

The software COANCESTRY 1.0.1.7 (Wang 2011) was used to estimate the pairwise relatedness (r_{xy}) between individual birds. A total of 873 birds were included in the analysis, 587 birds were DNA profiled and published in White et al. (2014), and an additional 286 DNA samples were collected across the breeding range of the species for pairwise relatedness examination. COANCESTRY implements seven methods to estimate the pairwise relatedness between individuals. These methods are: the triadic likelihood estimator (denoted as TrioML; Wang 2007); five moment estimators denoted as Wang (Wang 2002); LynchLi (Lynch 1988, Li et al. 1993); LynchRd (Lynch and Ritland 1999); Ritland (Ritland 1996); QuellerGt (Queller and Goodnight 1989); and a dyadic likelihood estimator (DyadML, Milligan 2003). The relatedness classifications for the genealogical relations were set at $r_{xy} = 0.5$ for parent-offspring and full siblings, $r_{xy} = 0.25$ for half siblings, and $r_{xy} = 0$ for unrelated individuals, with 1000 bootstraps to calculate 95% confidence intervals. For the purposes of this study, we report the genealogical relations of parent-offspring and full siblings from the 309 DNA samples collected at Coomallo Creek.

Distances between nest hollows used successively by the same breeding pair were calculated using the "show distance between waypoints" function of OziExplorer® software. The density of nest hollows in the study area was only calculated for the period 1972-1990 and 2009-2013 due to the fact that nest hollows were being located for the first time at a high rate during the first two years of the study (1970–1971).

Results

Between 1970 and 1990, observations of individually marked females provided data on nest hollow use by 92 breeding pairs, each making two or more breeding attempts. Pairs recorded making two or three breeding attempts provided 68.5% of the data. During that period one female was recorded making 12 breeding attempts (Table 1). Twenty of these breeding pairs had both birds individually marked. In all but one case the cockatoos remained paired with same partner, one pair being together for five years

Table 1. Number of breeding attempts made by individual females 1970–1990 and 2009–2013.

Number of breeding attempts made by individual females	2	3	4	5	6	7	8	9	10	11	12	Total
1970–90	45	18	15	7	2	1	1	0	2	0	1	92
2009–13	24	11	4	2	1	0	0	0	0	0	0	42

Table 2. The breeding outcome from, and location of, successive hollows used for breeding by 92 females 1970–1990 and 34 females 2009–2013. Figures in brackets are percentage of the total.

	# individuals	Successful; same hollow	Successful; different hollow; previous hollow occupied or unusable	Successful; different hollow; no information on hollow use	Unsuccessful; same hollow	Unsuccessful; different hollow	Total
1970–90	92	77 (45)	56 (33)	9 (5)	2 (1)	28 (16)	172
2009–13	34	28 (44)	23 (37)	4 (6)	1 (2)	7 (11)	63

(1972–1977), during which time they made seven breeding attempts. There was only one case of pair separation, where both partners were subsequently seen breeding with other partners. Between 2009 and 2013, DNA taken from nestlings provided data from 42 breeding pairs making two or more breeding attempts (Table 1). Most data (83.3%) were from females making two or three breeding attempts over the five year period, but one female made six attempts, one each year 2009-2013, and a second breeding attempt in 2013 after the first attempt failed. All attempts were with the same partner. One hundred and forty-two relationships between nestlings were established, ranging from two nestlings from the same breeding attempt to nestlings from six breeding attempts.

Data were obtained from 172 successive breeding attempts from the 92 pairs between 1970 and 1990, and 63 successive breeding attempts from 42 pairs between 2009 and 2013 (Table 2). One hundred and thirty-three (93.7%) successful breeding attempts between 1970 and 1990 were followed next season by the pair breeding in the same hollow or a different one if the previous one was not available. Between 2009 and 2013, 51 (92.7%) successful breeding attempts were followed next season by breeding in the same hollow or a different one if the previous one was not available. Between 1970 and 1990, 28 (93.3%) unsuccessful breeding attempts were followed by breeding attempts in a different hollow. Between 2009 and 2013, seven (87.5%) unsuccessful breeding attempts were followed by breeding attempts in a different hollow (Table 2). There was no difference (χ^2=1.21, d.f.= 4, p=0.88) between the relative rates of success or failure of breeding between the two phases of the study.

During the period 1970-1990, data on movements to different hollows between successive breeding attempts were obtained from 60 females that moved 94 times, and 2009-2013 from 30 females that moved 40 times (Fig. 2). The mean distance pairs moved between successive breeding attempts was 0.66±0.74km (n=94; range 0.02-4.05km) for the period between 1970 and 1990 and 1.25±1.31km (n=40, range 0.07 – 5.50km)

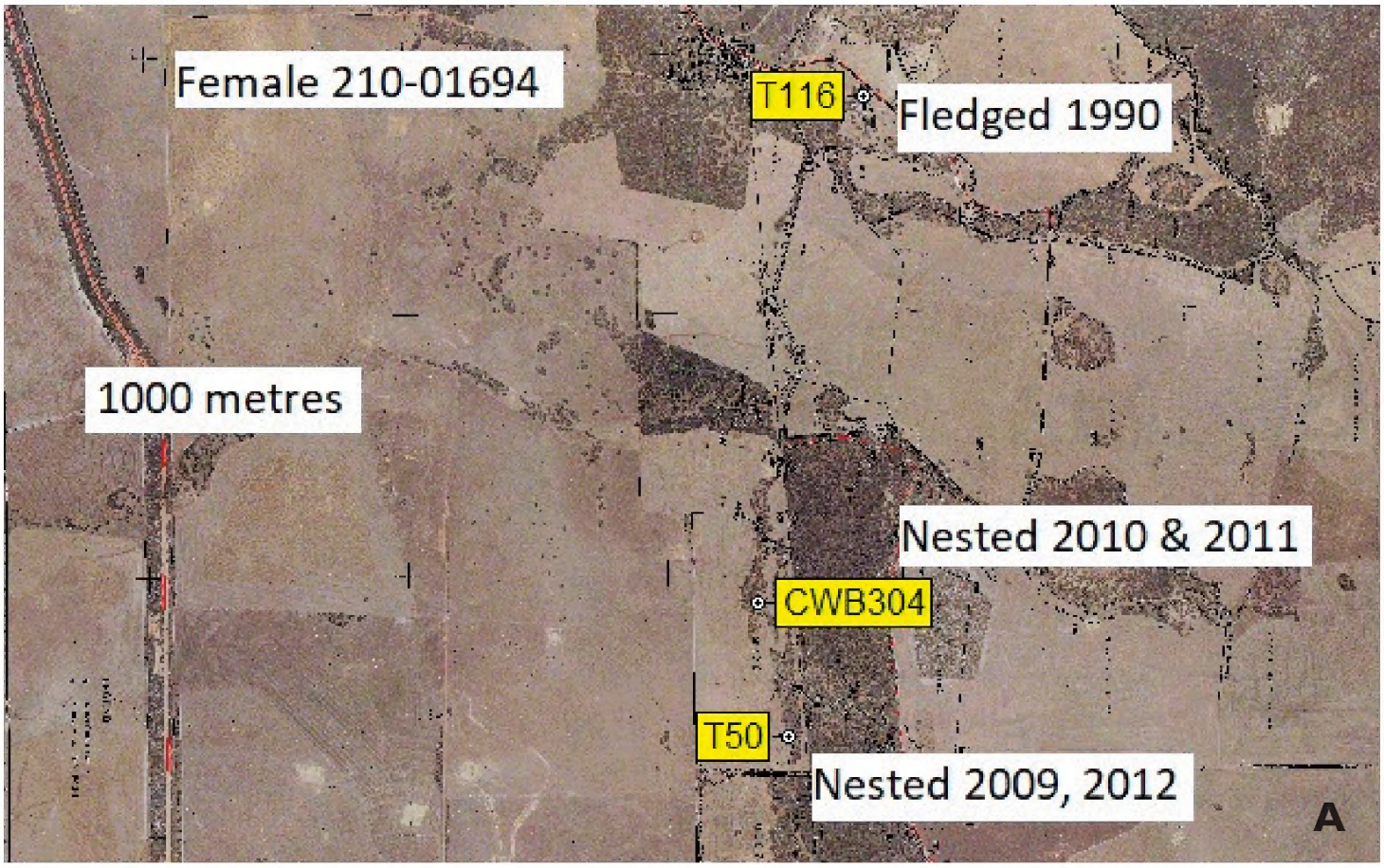

Figure 2. Movements between successive hollows by four females (two banded and two unbanded), confirmed by analysis of nestling DNA; **a** female 210-01694 fledged from T116 in 1990, and nested successfully each year from 2009 to 2012 in the hollows indicated **b** female 210-01876 was banded as an adult, and she nested successfully from 2009 to 2012 in the hollows indicated **c** unbanded Female A nested successfully in T43 from 2009 to 2012, was unsuccessful in T43 in her first breeding attempt in 2013, moved to CWB304 in which she was successful in her second breeding attempt that year **d** unbanded Female B nested successfully from 2009 to 2012 in the hollows indicated. The scale is indicated in the white rectangle with the width of the rectangle indicating the distance.

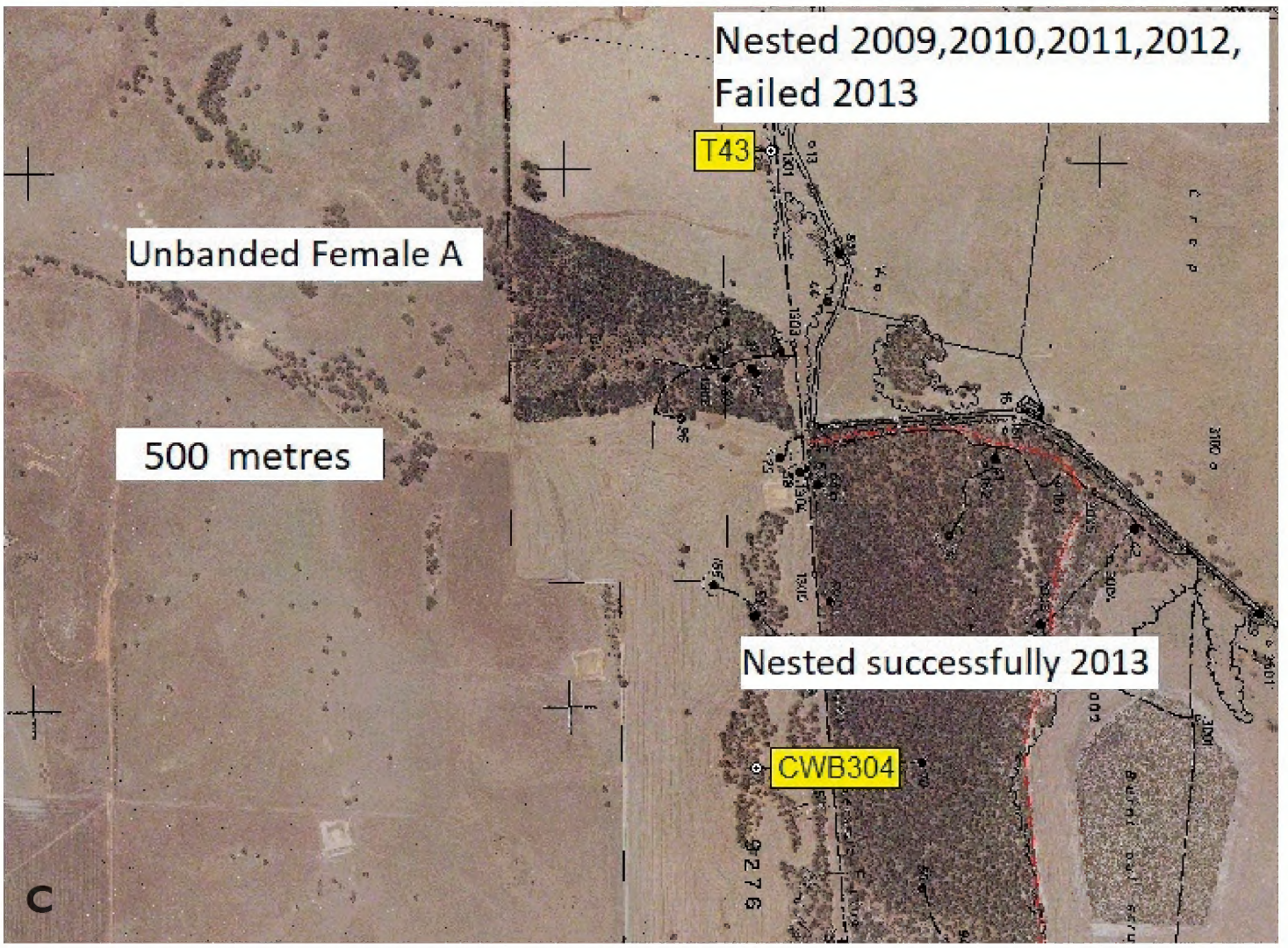

Figure 2. Continued.

between 2009 and 2013 (Table 3). There was a significant difference in the mean distances between successive nest hollows for the periods 1970-1990 and 2009-2013 (Mann-Whitney U-test, one-tailed: Z=-2.81, p=0.002). The distance between nesting hollows used in successive years increased despite the average density of nest hollows remaining

Table 3. Mean distance (km) between nest hollows used by Carnaby's Cockatoo in successive breeding attempts 1970–1990 and 2009–2013.

Years	Mean±s.d. distance moved (km)	Median distance moved (km)	Range	# individuals	# movements
1970–1990	0.66±0.74	0.36	0.02–4.05	60	94
2009–2013	1.25±1.31	0.56	0.07–5.50	30	40

Table 4. Density of nest hollows (km^{-2}) used by Carnaby 's Cockatoos 1972–1990 and 2009–2013.

Years	Mean±s.d. density of nest hollows (km-2)	Range
1972–1990	14.0±4.9	4.5–23.8
2009–2013	16.9±4.8	11.2–23.8

similar between the two phases of the study (Table 4; Mann-Whitney U-test, one-tailed: Z=-1.29, p=0.10).

For the period 2009-2013, DNA was obtained from both nestlings from 22 breeding attempts. Of these, six (27.3%) of the sibling pairs had different males fertilising the two eggs. Both nestlings fledged successfully in 13 cases where both eggs were fertilised by the same male (13/16 = 81.3%), and in four cases where the eggs were fertilised by different males (4/6 = 66.7%). Of the five cases where one or both of the nestlings died, both eggs were fertilised by the same male in three (60%) cases, and two (40%) by different males. The ages of three of the adult females for which there were DNA data on both nestlings from a single breeding attempt were known as the females were banded. Band number 210-03089 was at least 22 (she was banded as an adult), and both eggs were fertilised by the same male; 210-01876 was at least 26, 27 and 28, and the same male fertilised both eggs in all three clutches; and 210-01694 was 20, and the two eggs were fertilised by different males.

The average time between the laying of the two eggs in each case of siblings for which laying dates were known was 10.6±3.9 days (n = 15; range 5-17 days) where the same male fertilised both eggs, compared with 14.8±5.9 days (n = 5; range 9-24 days) where the second egg was fertilised by a different male. There was no significant difference in the mean interval (in days) between the laying of successive eggs in nests fathered by the same male and different males (t-test: t=-1.71, d.f.=17, p=0.053).

Discussion

Nest site fidelity, breeding site tenacity or breeding philopatry is a well-known phenomenon, demonstrated by many vertebrate species (Greenwood 1980). Among birds it is practiced by both migratory and sedentary species, from a wide range of taxa, both passerines and non-passerines, and in most species it is females that display breeding site fidelity (Greenwood 1980). Advantages accruing as a result of breeding site fidelity are

reduction in susceptibility to predation (Burger 1982) and other adverse conditions, such as micro-climatic impacts, and knowledge of rich food resources (Blancher and Robertson 1985, Ehrlich et al. 1994). In such species, breeding success in one season is often followed by use of the same site the next season (Blancher and Robertson 1985). There is tendency for breeding site fidelity to increase with age of individuals, based on the fact that older birds tend to be more experienced breeders, and therefore successful, so moving sites less frequently (McNicholl 1975). McNicholl (1975) pointed out that breeding site fidelity is strongly developed in highly stable habitats (e.g., cliff faces), but greatly reduced in highly unstable habitats (e.g., grasslands, mud beaches or bars in rivers).

Carnaby's Cockatoo breeds in highly stable habitats (*sensu* McNicholl), in that tree hollows are usually available for many years (Saunders et al. 2014b). So it is unsurprising that analysis of successive breeding attempts based on observations of individually marked females at Coomallo Creek between 1970 and 1990 confirmed breeding site fidelity, with 93.7% of successful breeding attempts being followed the next season by the birds breeding in the same hollow, or a hollow nearby if the hollow was in use when they returned to breed. Analysis of nestling DNA found the same result for the period between 2009 and 2013, with 92.7% of successful breeding attempts being followed the next season by the birds breeding in the same hollow, or a hollow nearby if the hollow was in use when they returned to breed. Such breeding site fidelity is shown by other species of Psittaciformes (Rowley 1990, Murphy et al. 2003, Berkunsky and Reboreda 2009). Analyses of data on hollow use following breeding failure from observations of individually marked females and nestling DNA confirmed Saunders' (1982) finding that after a failed breeding attempt, females usually moved to another hollow nearby for their next breeding attempt, either in the same breeding season or the next season.

Saunders (1982) established that when Carnaby's Cockatoo formed breeding pairs, in most cases they were monogamous, pairing for the life of one of the partners. This was based on observations of birds individually marked with patagial tags. Not only did they remain paired during the breeding season, but they remained together throughout the year (Saunders 1980). Analysis of nestling DNA confirmed this fidelity of breeding pairs from 2009 to 2013, but it also revealed extra-pair fertilizations. In 27.3% of the 22 cases where DNA samples were obtained from both nestlings from the same breeding attempt, the second egg was fertilised by a male not paired with the female. While these extra-pair fertilizations were surprising in light of observations of individually marked breeding Carnaby's Cockatoo, this extra-pair fertilization is not uncommon in birds (Petrie and Kempenaers 1998, Forstmeier et al. 2011), and mammals (Crawford et al. 2008). Extra-pair fertilization has been recorded at varying rates in some larger parrot and cockatoo species, for example: 9% extra-pair fertilization in nests of Blue and Yellow Macaw *Ara ararauna* (Caparroz et al. 2011); 20% in nests of Red-tailed Amazona *Amazona brasiliensis* (Fernandes 2015); and 40% in nests of Monk Parakeet *Myiopsitta monachus* (Martinez et al. 2013). In parrot species with multi-male breeding systems, extra-pair fertilizations have been suggested in the Echo Parakeet *Psittacula eques* (Taylor and Parkin 2009), confirmed in Eclectus Parrot *Eclectus roratus* (Heinsohn et al. 2007), and confirmed in 100% of nests of Vasa Parrot *Caracopsis vasa* (Ek-

strom et al. 2007). However, in other species such as the Burrowing Parrot *Cyanoliseus patagonus* (Masello et al. 2002) and Crimson Rosella (*Platycercus elegans*; Eastwood et al. 2018) there is no evidence of extra-pair fertilization.

The chances of observing extra-pair copulations of individually marked birds were low. While many breeding females were individually marked with patagial tags and/or leg bands, far fewer males were individually marked. While copulations were observed, those few cases where both sexes were identified were all with known mates, reinforcing the conclusion of monogamy. In 2010 a documentary about the breeding of Carnaby's Cockatoo was filmed (http://www.abc.net.au/tv/guide/abc1/201203/programs/NH1001W001D2012-03-13T203229.htm accessed 5 April 2018). The documentary was based on one breeding pair, and after the first egg was laid, film was taken of the female leaving the hollow when her unbanded mate was not around. There were two adult males nearby, and both immediately started courtship displays towards the female. One of the males had a leg band, and that could have only been placed on the bird seven years previously or longer. Earlier observations and this film clearly indicate that males will readily court breeding females other than their mates (Saunders 1979), and analysis of nestling DNA indicates that females will accept copulation from males other than their mate. It is highly unlikely that the first egg would result from copulation with a male other than the mate. This is because the pair remain together from the time they return to the breeding area, select and prepare their nesting hollow, and the first egg is laid. At that time the female incubates the egg, and depends on the male for food (Saunders 1982), and males are aggressive to other males approaching their mate (Saunders 1979). During incubation, and until the older nestling is about three weeks old, the female remains either in the hollow or around the nest tree. The male is around the nest tree at dawn, mid-morning when he feeds the female, and again when he feeds her at dusk (Saunders 1982). On occasions the female may be out of the hollow when the mate is not around. At this time extra-pair copulations are possible. The second egg is laid on average eight days after the first, but may be laid up to 24 days later (Saunders 1982, Saunders et al. 2014a). At Coomallo Creek, both nestlings have fledged from hollows when there was 24 days difference in age, with nestlings growing at the same rate; i.e., the younger nestling does not grow faster than the older in order to fledge at the same time. These differences in time between the eggs provide ample opportunity for extra-pair copulations. The limited data available from analysis of nestling DNA indicate that older, more experienced females may indulge in extra-pair copulations, and that second nestlings, the result of such copulations may fledge along with the older nestling, the result of copulation with the male of the pair. These data came from banded females, however nothing is known about such rates in younger breeding females.

Who were the males responsible for these extra-pair copulations? Of the six cases known to us, in three cases, there were no data indicating the second nestling was a half-sibling to any other nestlings from the area that breeding season for whom we had DNA. In each of the other three cases (one in 2009, 2010 and 2012), there was a nestling from three other breeding pairs that indicated a half-sibling relationship. The average distance between any of these hollows and the hollow with the extra-pair copulations was 2.8±1.2km (range 1.7–5.2km). If it was one of the males producing the

half-siblings, they weren't neighbours. Without DNA from the males, unfortunately we cannot identify any of the males responsible.

The conservation implications for such extra-pair fertilizations in Carnaby's Cockatoo are difficult to discern. Extra-pair fertilizations within local breeding populations would result in broader genetic diversity within breeding populations than would result from purely monogamous pairings.

Conclusions

Comparisons of results obtained from long-term studies based observations of individually marked Carnaby's Cockatoo breeding adults, and those from analyses of DNA from Carnaby's Cockatoo nestlings showed that analyses of DNA are useful for establishing stability of breeding pairs and breeding site fidelity. Analyses of nestling DNA demonstrated that aspects of mating behaviour that would be extremely difficult to show using observations of individually marked adults could be revealed by studies of DNA. Analyses of DNA from nestlings over subsequent breeding seasons would be necessary to investigate the identity and ages of the adults involved in extra-pair copulations, and provide evidence of the advantages to the species of breeding pair infidelity.

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